Glucose cloning protocol

First step:

Primers arrived => Dilute them as described from the compeny

Seconed step:

1. 2-stage PCR reaction for:

variant	F-primer	R-primer	Tm1	Tm2
1	Glu_BA_For	Glu_AA_Rev	48.6	65.7
2	Glu_BB_For	Glu_AB_Rev	55.6	68.3
3	Glu_BB_For	Glu_AC_Rev	55.6	68.3
4	Glu_BC_For	Glu_AD_Rev	55.6	66.4
5	Glu_BD_For	Glu_AB_Rev	55.6	68.3
6	Glu_BE_For	Glu_AE_Rev	48.6	67
7	Glu_BF_For	Glu_AF_Rev	55.6	66.9
8	Glu_BG_For	Glu_AF_Rev	55.6	66.2
control	Glu_BD_For	Glu_AB_Rev	55.6	68.3

Set up reaction:

Component	**Volume**	**control**
5x PCR Buffer	10 µl	10 µl
dNTPs (10mM)	1 µl	1 µl
Forward primer [10 µM]	2.5 µl	2.5µl
Reverse primer [10 µM]	2.5 µl	2.5µl
Template DNA - Tar2 CM (2- 10 ng)	1 µl	-
Phusion DNA Polymerase	0.5 µl	0.5
UPW	32.5 µl	33.5 µl
Total	50	50

PCR program:

cycles	**Step**	**Temperature**	**Time**
1	Initial denaturation	98	30 sec
10	Denaturation	98	10 sec
	Annealing	grad of 48.6-55.6	30 sec
	Extension	72	2 min
20	Denaturation	98	10 sec
	Annealing	grad of 65.7-68.3	30 sec
	Extension	72	2 min

1	Final extension	72	10 min
1	Hold	4	hold

30 cycles

2. Gel electrophoresis (p.10-12) - 1% agar (500 bp to 10 kb)
   5 µl of ladder
   4 µl of sample + 1 µl dye

ladder	1	2	3	4	5	6	7	8	control

Expected length-

THE WHOLE TAR- 3930bp

3. Purification of PCR product (Equal volume = 45 µl)

variant	conc.(ng/µl)	260/280	260/230
1			
2			
3			
4			
5			
6			
7			
8			

4. DpnI for DNA (in order to digest methylated plasmid)
   Set up reaction:

component	Volume(ul)
DNA	30
10X CutSmart Buffer	4
DpnI	1
UPW	5
Total	40ul

Incubate for 1 hour in 37°C

5. Purification of DpnI product

variant	conc.(ng/µl)	260/280	260/230
1			
2			
3			
4			
5			
6			
7			
8			

6. Phosphorylation
   Set up reaction:

component	Volume(µl)
T4 DNA ligase buffer	2.5 µl
T4 kinase (PNK)	1 µl
DNA	X~100 ng= 3µl
PEG 4000	2.5 µl (5%)
MB H2O	19-X µl
Total	**25** µl

Incubate for 30 minutes in 37°C
Heat inactivation prior to ligation for 20 minutes in 65°C
Cool on ice for 10 min

7. Self Ligation:
   Add 1 µl of T4 DNA ligase to phosphorylation reaction tube
   Incubate for 2 hours in 30°C

8. Transformation to UU
9. Starters + mini prep
10. Sequencing
11. Chemotaxis checks: Swarming assay, microscope